Generation and Characterization of Liposomal Microcapsules for Co-delivery of Hydrophilic and Lipophilic Bioactives in Food

Abstract:

In this report, supercritical CO_2 was used, as a green platform, to produce liposomes without using organic solvents and cholesterol. Different parameters such as pressure of the vessel, temperature of the nozzle, and nozzle diameter, were changed in order to study their effects on the shape, size, uniformity, and stability of the resulting liposomes. It was found that our method is a versatile technique to tune the structure of liposomes. The results showed that when the pressure of the vessel and diameter of the nozzle increase, the concentration and encapsulation efficiency (EE) of the liposomes increases. The increase of nozzle temperature from 70 °C to 85 °C, improves the EE due to the fact that the Joule Thompson effect reduces. However, when temperature of the nozzle increases more to 100 °, the EE decreases because the solubility of lecithin is inversely proportional to the temperature.

Keywords: Supercritical CO_2, Liposomes, Green Technique, Nozzle Design

1. Introduction:

Liposomal microcapsules are colloidal vesicles consist of spherical phospholipid bilayers surrounding a water phase interior compartment [1]. Liposomal microencapsulation has received much attention due to their wide range of applications such as food science, pharmaceutical, and cosmetics [2-5]. The delivery of these vesicles can be accurately engineered. Liposomes can encapsulate both hydrophilic and hydrophobic cargos due to their amphiphilic nature [6, 7]. The surface chemistry of the liposomes can be modified such that external stimuli such as temperature, pH, light, and osmotic pressure shock, can trigger the liposome release [8-10]. Additionally, liposomes show higher stability, in environments with high water activity, than microcapsules made of conventional approaches, such as spray drying or extrusion. In terms of structure, they mimic the lipid vesicles exist naturally in living organisms. The rigidity, fluidity, and

permeability of phospholipid bilayer can be controlled by using cholesterol, different surfactants, or carbohydrates. Liposomes can be categorized based on their structures into three types: unilammelar Vesicles (ULV), multilamellar vesicles (MLV), and multivesicular vesicles (MVV) [1].

In food science, liposomes were used to encapsulate antioxidants, nutrients, enzymes, antimicrobials, and additives [4, 11, 12]. They are used to control delivery of functional components such as vitamins, enzymes, and flavors in different food applications. Solubility, stability, and bioactivity of foods can be enhanced through lipid-based micro/nano encapsulation [13]. It has been around two decades that the liposomes applications in dairy products are studied. It is a good approach to use liposomes for producing low calorie and reduced fat products due to their high dispersity in water. Additionally, liposomes can be used to prevent oxidation, make off flavors, decrease energy density of food products, and control their release. For instance, Tsai and Rizvi showed simultaneous encapsulation of hydrophobic and hydrophilic bioactives, Vitamin C and Vitamin E, respectively, in the integrated liposomes [7]. They utilized supercritical fluids and studied different parameters such as pressure and temperature on the characteristics of the resulting liposomes.

Different approaches have been used to make liposomes. The conventional liposome production methods include Thin film hydration (TFH, the Bangham method) and reverse phase evaporation vesicles (REV). However, the conventional liposome production methods are inevitably dependent on using toxic organic solvents, such as chloroform, which make the resulting liposomes unsuitable candidates for most of the applications. Some methods have been used to remove the organic solvent residues, but they are expensive and difficult to scale-up. In order to avoid the use of organic solvents, alternative techniques were applied such as rapid expansion of supercritical solvent (RESS), and supercritical reverse phase evaporation (scRPE) [1, 14, 15]. In RESS and scRPE, supercritical CO_2 (SC-CO_2) is used as an alternative solvent, instead of organic solvents, in the process of liposome production. Supercritical fluids (SCFs), in particular SC-CO_2, has received much attention as a potential alternative to organic solvents. SCFs have liquid like density and can easily dissolve polar and non-polar materials. The solubility of different solutes into SCFs can be easily tuned through changing different

parameters such as temperature and pressure of SCFs. Additionally, there is no need to do post-process for sterilizing the products due to the fact that SC-CO_2 provides a sterile production environment [2, 16].

In RESS technique, a supercritical solution is depressurized by passing through a nozzle. When the pressure drops, a transition occurs for the solvent from the supercritical state to gas state. Because the solvating power of the solvent in the gas state is significantly lower, the dissolved solute nucleates. Wen et al. used this technique to make the liposomal microencapsulation of rose essential oil, and they used three surfactants, Tween 80, deoxycholic acid sodium (DAS), and Poloxamer 188, to study the stability of the resulting liposomes [17]. In scRPE approach, an organic solvent is used to dissolve the phospholipid. Then, the organic solvent is replaced by the SC-CO_2 to produce homogenous liposomal particles [18-20].

Although many advantages, using SC-CO_2 has some limitations for producing liposomes at this point. The organic solvents are still used as a cosolvent in order to improve the performance of the technique. Additionally, it is required to increase the pressure of CO_2 to obtain the super critical state. Therefore, the selection of the cargo solution would be limited to the ones that can withstand the high pressure and force exerted from SC-CO_2 in the mixing chamber. Here, we produced different types of liposomes without using an organic solvent. In some studies, the cargo solution is introduced into the mixing chamber with a pump, whereas we do not use an external energy to pump the cargo solution into the mixing chamber. We show that our method can be used to produce liposomes with different sizes and types. Although in most of the studies, cholesterol is used to strengthen the wall of the liposomes, we proved the fact that our method has the ability to produce liposomes with high stability without using cholesterol.

2. Materials and Method

2.1 Materials

Carbon dioxide (CO_2) with 99.99% minimum purity was purchased from Airgas (Ithaca, NY, USA). Tris(hydroxylmethyl) aminomethane (TRIS) was provided from Bio-Rad (Hercules, CA, USA). Nile Red, dialysis tube (Pur-A-Lyzer Midi dialysis tubes), and

hexokinase assay kit were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The carrier of the liposomes was made of SOLEC FP 40, which is a lecithin with 39% of phosphatidylcholine (PC), was provided from Solae company.

2.2. Preparation of the aqueous cargo solution, carrier, and fluorescent dye

0.02 M TRIS buffer was prepared and its pH was set at 7.4 using Hydrochloric Acid (HCl). Cargo solution was made of 0.2 M D-(+)-glucose solution in TRIS buffer. Vessel was loaded with 5 g of SOLEC FP 40. The vessel has a mixer that help the system reach the equilibrium in a shorter amount of time. The carrier part of the liposomes (lecithin) was stained using Nile Red. The concentration of 0.02 wt% Nile Red was made by adding 4 mg of Nile Red to 20 mL of absolute ethanol.

2.3 Charaterization

2.3.1 Imaging

10 µL of Nile Red solution was mildly mixed with glucose-encapsulated liposomal particles in order to stain the phospholipid bilayer. After 1 min of mixing, we centrifuged the sample for 5 minutes to separate the stained liposomes from the free stains. 40 µL of the samples from the pellet part was used for both regular and confocal laser scanning microscope (Leica Upright DMRE-7 confocal microscope, Buffalo Grove, IL, USA, fitted with the 10× ocular lens and the 100× oil-phase objective lens) in order to study the inner structures of the liposomes. The fluorescence emission spectrum of Nile Red was set at 558 to 635 nm. SEM imaging was done with JCM-6000 NeoScope Benchtop SEM. For Cro-SEM imaging, a Thermo Fisher Strata 400S was used. For the sample preparation in Cryo-SEM imaging, the samples were rapidly cooled using liquid Nitrogen and transferred onto the SEM chamber under vacuum condition.

2.3.2 Encapsulation efficiency

Our method for measuring the encapsulation efficiency (EE) of the D-(+)-glucose loaded liposome was based on Manosroi et al [21]. Briefly, 500 µL of the liposome solution (liposome stored in TRIS Buffer) was loaded in a dialysis tube (Pur-A-Lyzer Midi dialysis tubes) and dialyzed against 500 µL of TRIS buffer with the concentration of 0.02 M and

pH of 7.4 for 12 hours at the temperature of 4 °C to make sure the free glucose (un-encapsulated) passes through the dialysis tube. After 12 hours, in order to rupture the liposomes, 880 µL of 4% Triton solution was mixed with 120 µL of the purified liposomes and left to settle for at least 3 minutes. 20 µL of the ruptured liposomes was reacted with the hexokinase and measured the absorption at 340 nm. The content of encapsulated glucose in the liposomes was calculated using the equation in hexokinase assay kit. Then, below equation was used to calculate the encapsulation efficiency of the liposomes:

$$EE\ (\%) = (Cargo\ content\ in\ purified\ liposomes / total\ cargo\ content\ in\ the\ initial\ cargo\ solution) \times 100$$

2.3 Method: Liposome production using SC-CO_2

We developed a high-pressure eductor nozzle-based RESS system for producing liposomes using SC-CO_2 without using an organic solvent (**Figure 1**). When the system starts, high pressure CO_2, containing the carrier, releases and accelerates by passing through a converging-diverging nozzle and its velocity increases.

Figure 1 (a) shows the steps for generating SC- CO_2. A cooler is used to cool CO_2 down to liquid state. Then, it is pumped using a high-pressure pump, preheated, and finally kept in a high pressure mixing vessel. In the vessel, the carrier (lecithin here) is dissolved into the SC-CO_2. As shown in **Figure 1 (b)**, the solution is injected into the eductor nozzle. Because the velocity significantly increases at the *vena contracta*, pressure drops to below atmospheric pressure (Bernoulli equation). The hydrophilic cargo solution is introduced into the educator nozzle due to the vacuum created at the low-pressure zone. The solubility of lecithin in CO_2 significantly decreases after the nozzle due to the pressure drop. Then, carrier material rearranges itself and wraps around the hydrophilic cargo. Interaction between the cargo solution and the carrier material forms the liposomes, which are collected in a buffer solution.

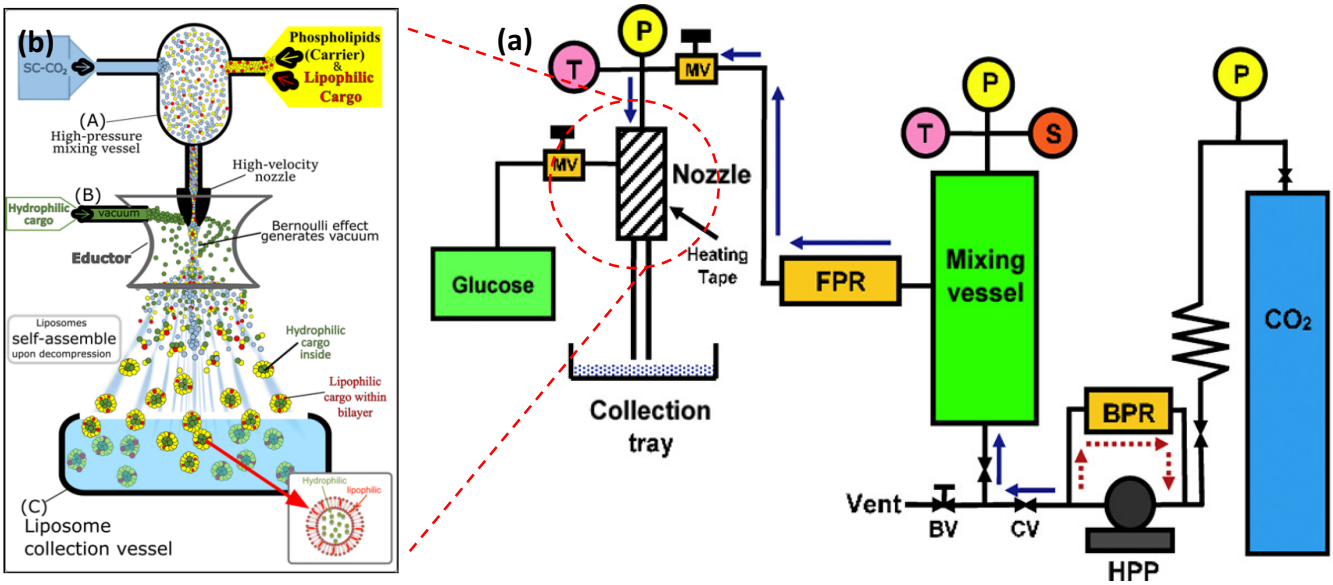

Figure 1. *Schematic of liposome production using SC-CO_2. (a) Thermodynamic cycle used to obtain supercritical state CO_2: BPR: back pressure regulator, HPP: high pressure pump, FPR: forward pressure regulator, MV: metering valve, BV: ball valve; CV: check valve; S: safety valve P: pressure indicators, T: temperature indicators. (b) Encapsulation process with more details. The low-pressure zone was used to load the eductor with cargo solution.*

3. Results and Discussions:

The process of producing liposomes with our technique has different parameters, which can play role on the structure, size, uniformity, and stability of the resulting liposomes. In this work, we studied the effect of some of the parameters. **Figure 2** shows liposomes made at different vessel pressures and nozzle temperatures. When the vessel pressure increases, the solubility of lecithin in SC-CO_2 increases, which results in higher concentration of liposomes. Additionally, higher amount of lecithin can make liposomes with higher surface areas, which might be the reason that the size of the liposomes decreases from 11 MPa to 14 MPa. When CO_2 passes through the nozzle and pressure drops, the temperature of CO_2 decreases significantly due to the Joule Thompson effect. Increasing the temperature of the nozzle can help keep the product temperature higher

than melting point of lecithin (40 °C). That might be the reason that the increase of the nozzle temperature, can make the liposome size more uniform.

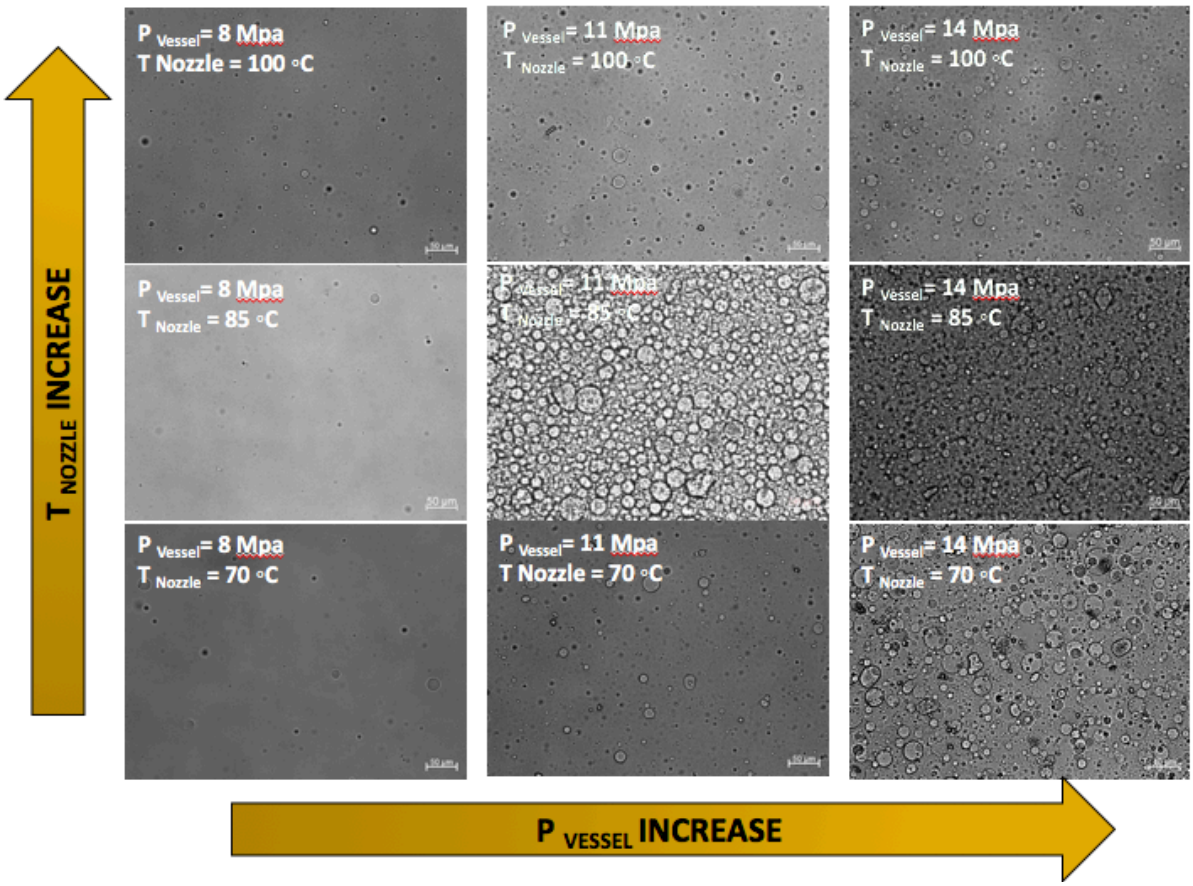

Figure 2. *Liposomal microcapsules made at different vessel pressures and nozzle temperatures*

In order to study the particles and make sure that they are liposomes, the carrier was stained using Nile Red (protocol provided). We used different nozzles with the orifice diameters of 0.5 mm, 1.0 mm, and 1.5 mm. **Figure 3** shows fluorescent images of the liposomes made by using different nozzles. This figure demonstrates that when nozzle diameter increases to 1.5 mm, almost all of the products are liposomes and the phospholipid bilayer is clear in the images. We did one second blast for all three-sample production. The concentration of the liposomes increases when larger nozzle diameter is used. That was expected because the flow rate is proportional to the nozzle diameter and

by increasing the nozzle size, more lecithin passes through the nozzle which increases the possibility of liposome formation.

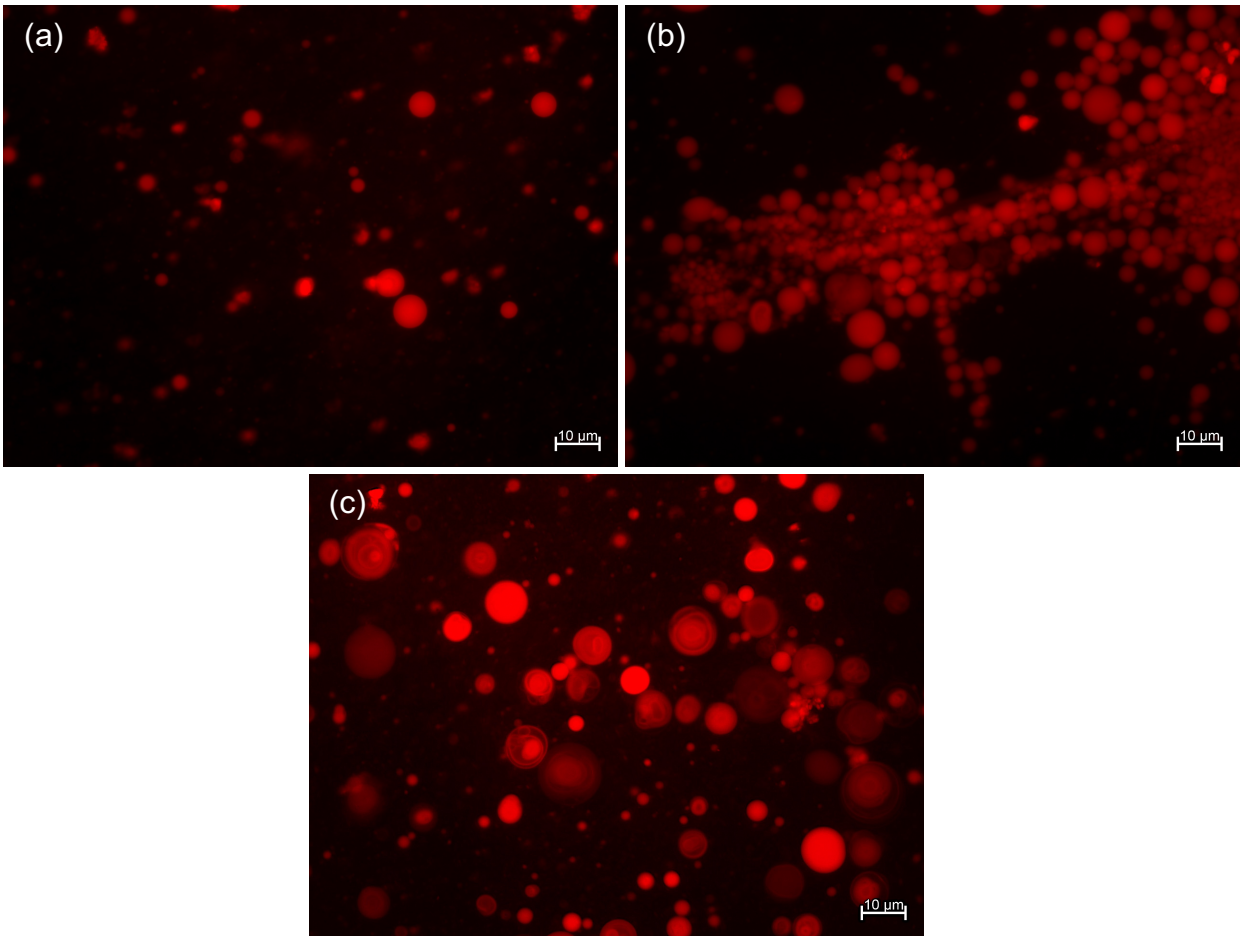

Figure 3. *Fluorescent images of the liposomes generated using nozzles with (a) 0.5 mm, (b) 1.0 mm, and (c) 1.5 mm.*

Figure 4 shows the glucose-encapsulated liposomes after one month, made by 1.5 mm nozzle, using confocal laser scanning microscopy (CLSM) at two different z-stacks. This images clearly show that different types of liposomes, which are ULV, MLV, and MVV, can be produced using SC-CO_2. This figure demonstrates that the liposomes have high stability and can keep their structures after one month. To best of our knowledge, in other studies, organic solvents are used, as the main solvent or cosolvent, in order to increase the solubility of the carrier. Additionally, cholesterol is typically used to strengthen the wall

of the liposomes and increase their stability. In our method, we did not use any of them and we obtain high concentration of the products with high stability.

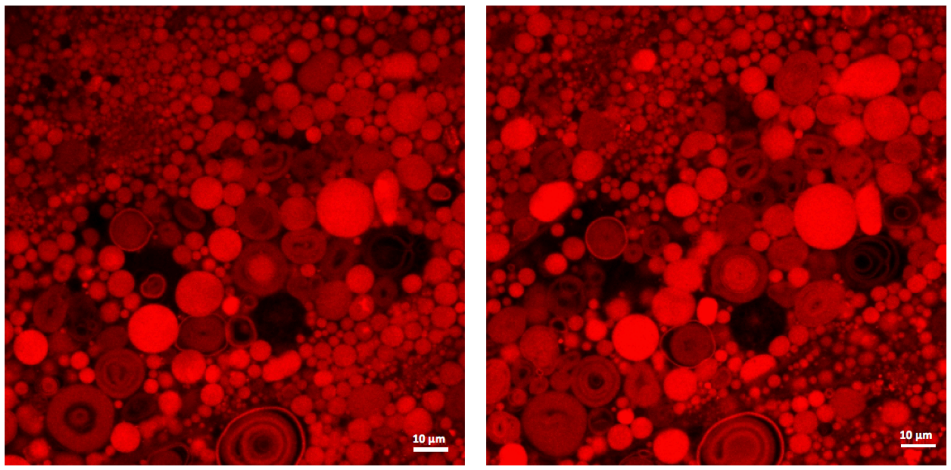

Figure 4 *CLSM visualization of the liposomes produced using 1.5 mm nozzle at* P_{vessel}*= 14 MPa.*

Figure 5 shows SEM images of the liposomes made by SC-CO_2 with a 1 mm nozzle at P_{vessel}= 14 MPa. In some of them, the liposomes had a tendency to coalesce and form large particles (**Figure 5 (a_1-a_2)**). Although they coalesce, it is clear that there had been a water phase inside of the particles, which evaporated and the liposomes coalesced. **Figure 6** shows Cryo-SEM images of a liposome made using SC-CO_2, 1.5 mm nozzle at P_{vessel}= 14 MPs, which was cut using ion beam two times in order to observe two sections of the liposomes. This figure shows that the inner part of the liposome is not solid and it has some empty regions, which might be the location of the water phase inside of the structure of the liposome. Based on the current SEM images, it is difficult to conclude anything about the structure of the liposomes. In order to improve the quality of the SEM imaging, higher concentration of liposomes is needed and the liposomes should be separated from the free lecithin floating in the buffer solution.

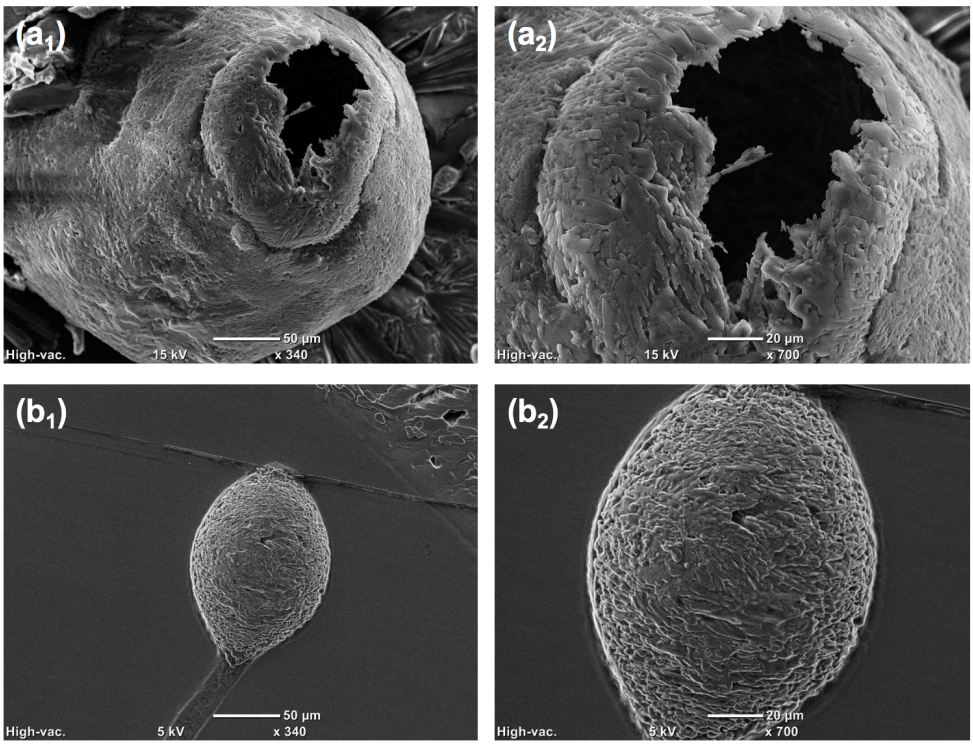

Figure 5. *SEM image of liposomes made by 1 mm nozzle at P_{vessel}= 14 MPa.*

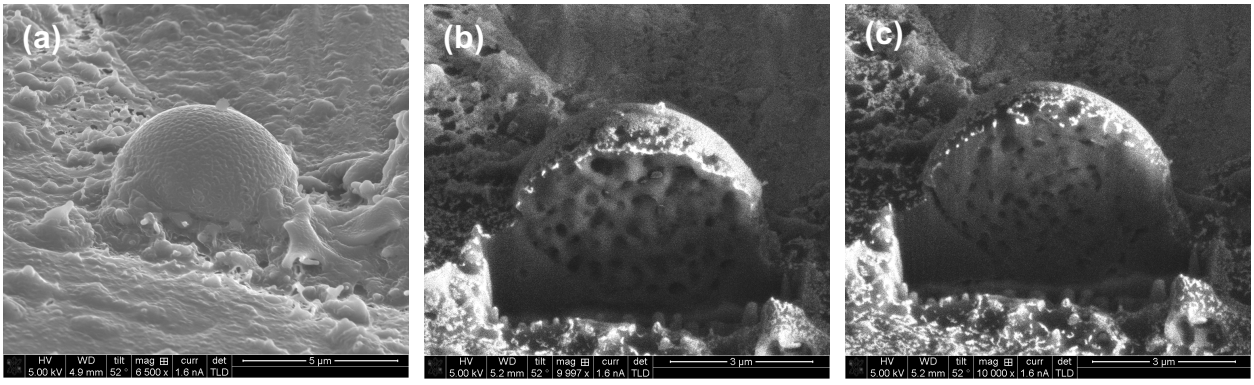

Figure 6. *Cryo-SEM image of liposomes made with 1.5 mm nozzle at P_{vessel}= 14 MPa: (a) the morphology of the outer layer of liposomes, (b) cross-section of the liposomes cut using ion-beam, (c) another section of the liposome cut by ion beam.*

Table 1 shows the encapsulation efficiency (%) of the liposomes produced at different vessel pressures (8 MPa, 11 MPa, and 14 MPa), nozzle temperatures (70 ºC, 85 ºC, and 100 ºC), and made by nozzles with different orifice diameters (0.5 mm, 1.0 mm, and 1.5 mm), which was calculated with hexokinase assay kit. This table shows that

liposomes with encapsulation efficiency (%) in the range of 0.89%-41.66% can be produced. When the pressure increases, EE improves for all of the cases. That was expected because when the pressure of the vessel increases, the solubility of lecithin in SC-CO_2 increases. This factor causes that most of the cargo solution, introduced into the nozzle, is coated and liposomes with different structures are formed.

Nozzle diameter plays a significant role on encapsulation efficiency of the liposomes. When nozzle with larger orifice diameter is used, the encapsulation efficiency improves significantly. In this condition, when larger nozzle is used, the supercritical flow rate increases because the flow rate is proportional to the resistance in the flow and nozzle with larger diameters make lower resistance. When higher amount of carrier material is introduced in the eductor, the concentration of the liposomes increases, which results in obtaining higher encapsulation efficiency.

Table 1 *Encapsulation Efficiency (%) of the liposome produced at different vessel pressures, nozzle temperatures, and nozzle sizes*

Temperature = 70 ºC			
Pressure	**Nozzle Diameter**		
	0.5 mm	**1.0 mm**	**1.5 mm**
8mpa	0.89 ±0.63 b,C	6.65±1.09c,B	17.29± 1.09b,A
11mpa	3.10 ±0.63 a,C	11.97±1.09b,B	20.83±0.63a,A
14mpa	4.43 ±0.63 a,C	18.17±0.63a,B	22.60±1.08a,A
Temperature = 85 ºC			
Pressure	**Nozzle Diameter**		
	0.5 mm	**1.0 mm**	**1.5 mm**
8mpa	5.32±1.09c,B	7.09±0.63c,B	23.49±0.63c,A
11mpa	12.85±0.63b,B	14.18±3.13b,B	31.91±1.09b,A
14mpa	19.95±1.09a,C	27.48±2.26a,B	41.66±1.25a,A
Temperature = 100 ºC			
Pressure			
	0.5 mm	**1.0 mm**	**1.5 mm**
8mpa	1.77±0.63c,C	7.09±2.26c,B	18.17±0.63c,A
11mpa	8.86±0.63b,C	13.30±2.17b,B	25.26±1.09b,A
14mpa	18.62±1.09a,C	22.16±1.25a,B	28.81±0.63a,A

In addition to pressure and nozzle diameter, nozzle temperature is another important parameter that can change the encapsulation efficiency. The results show that when the nozzle temperature increases from 70 ºC to 85 ºC, EE increases and then, by increasing the temperature up to 100 ºC, the EE decreases for all of the cases. The reason might be

due to the fact that when the temperature of the nozzle increases, the Joule Thompson effect decreases, which means that higher temperature prevents that the product temperature becomes lower than melting point of the liposomes. This parameter help increases the possibility of liposome formation. When the nozzle temperature increases more, however, the solvating power of SC-CO_2 decreases, which means some of the lecithin, already dissolved in SC-CO_2 in the vessel, precipitates. This decreases the liposome formation possibility, which results in obtaining lower EE.

Conclusion:

In this study, liposomal microcapsules were made using supercritical CO_2 without using any organic solvent and cholesterol. In this platform, it was shown that the morphology, shape, concentration, and performance of the liposome in encapsulation water phase materials can be tuned by changing different parameters such as pressure of the vessel, temperature of nozzle, the diameter of the nozzle orifice. When the pressure of the nozzle increases, the solvating power of SC-CO_2 increases, more lecithin dissolves in SC-CO_2, which results in higher concentration of liposomes and higher encapsulation efficiency. When a nozzle with larger orifice diameter is used, SC-CO_2 will have less resistance. As a result, the flow rate of the SC-CO_2 increases, and more carrier material will pass through the nozzle. That might be the reason that the encapsulation efficiency significantly increases as the nozzle diameter increases. Temperature of the nozzle can affect the structure of the liposomes as well. When the temperature increases, the liposomes become more uniform and their encapsulation efficiency increases due to the fact that the effect of Joule Thompson reduces. However, when the temperature increases more, the EE reduces, which might be due to the fact that the solubility of the lecithin in SC-CO_2 decreases as the temperature increases.

Future work:

- There are other factors that can potentially play role in changing the structure of the liposomes, such as vessel temperature, cargo suction volume, cargo temperature, nozzle type, etc, which can be studied in order to have a comprehensive knowledge about the important parameters on liposome formation.

- The experiment needs to be normalized such that in each blast, we have an equal amount of SC-CO_2 passes through the nozzle regardless of the nozzle diameter. For our previous results, we kept the blast time constant. However, its drawback is that when we change the nozzle, different volume of SC-CO_2 passes through the nozzle.
- In order to improve the uniformity and encapsulation efficiency, the nozzle design needs to be modified. In this regard, different approaches are considered. All of our current nozzles have zero-degree spray angles. For this type of configuration, the cargo introduction tube has to be exactly in front of orifice hole in order to have a decent amount of mixing. We can study the effect of different spray angles to optimize the best angle in order to make sure that the cargo solution is atomized and mixed with the carrier material (**Figure 7**).

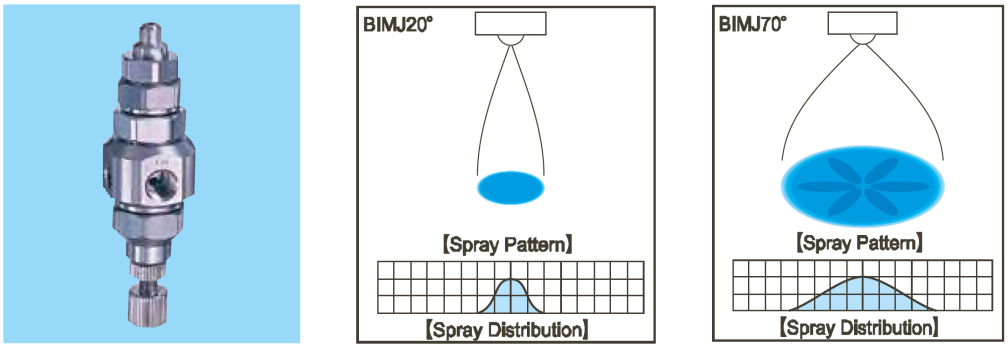

Figure 7*. Schematic of the eductor that can potentially enhance the mixing between the cargo solution and the carrier. Nozzle with different angles can be used in order to obtain liposomes with high uniformity and EE.*

- The shortcoming of using the above eductor is the angle between the cargo introduction part and the SC-CO_2 flow. If the angle between these two flows is not zero, or close to zero, there is always a possibility of back flow due to the high-pressure range that we use in our experiment (2000-3000 psi). In order to alleviate this issue, we can use a pipe to make sure that the angle between the two flows is zero. The other approach is to design a new eductor such that the angle between

to fluids is zero and the fluids have enough time to mix with each other. Figure 8 shows a schematic of a converging-divering nozzle embedded in a chamber. After the nozzle, there is a converging diverging diffuser that can help improve the mixing between the cargo and carrier solutions. The angle between the two fluids is zero degree to minimize the effects of back flow. The length of the diffuser can be changed in order to optimize the best mixing conditions. The process of liposome formation will be simulated using COMSOL Multiphysics in order to optimize the geometry and obtain the highest mixing and vacuum pressure. Then, it will be made using 3D printing approach

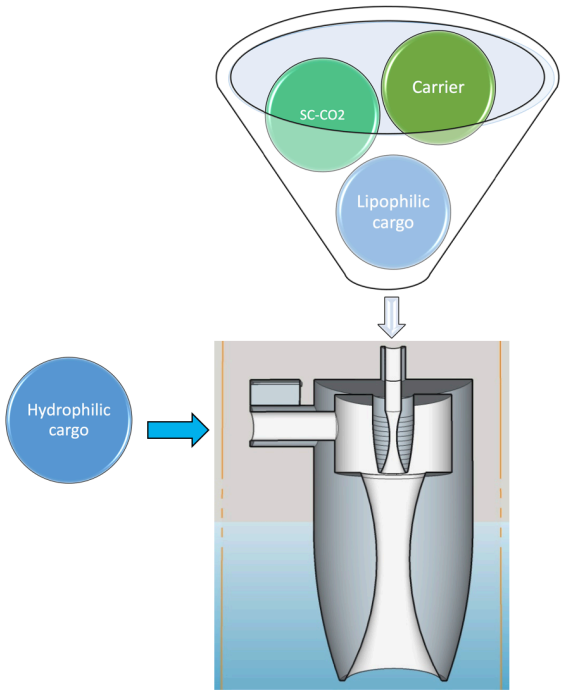

Figure 8. *Schematic of an eductor that can potentially improve the uniformity and encapsulation efficiency of the liposomes with minimum back flow effects.*

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